ARCEUTHOBIUM GILLII AND *A. NIGRUM* (VISCACEAE) REVISITED: DISTRIBUTION, MORPHOLOGY, AND RDNA-ITS ANALYSIS

Shawn C. Kenaley

Department of Plant Pathology
and Plant-Microbe Biology
Cornell University
Ithaca, New York 14853, U.S.A.
sck26@cornell.edu

Robert L. Mathiasen

School of Forestry
Northern Arizona University
Flagstaff, Arizona 86011, U.S.A.
Robert.Mathiasen@nau.edu

ABSTRACT

Additional morphological, phenological, and molecular data were obtained for *Arceuthobium gillii* and *A. nigrum* from throughout their geographic distributions in northern Mexico and southern Arizona. Multivariate analysis of variance (MANOVA) and discriminant function analysis demonstrated that *A. gillii* and *A. nigrum* are distinguishable morphologically. Discriminant function analysis also indicated that the dimensions of the third internode (length and width) and basal diameter of female and male plants contributed most to the classification and predication of species. Significant differences in plant height (female and male) as well as flower and fruit dimensions were also evident between these dwarf mistletoes, *A. nigrum* having larger dimensions for each of these characters. Although *A. nigrum* purportedly flowers twice annually (spring and fall), our observations indicated that the period of anthesis for this mistletoe occurs only once annually—fall to early winter. In contrast, *A. gillii* flowers in the spring; thereby, suggesting that these taxa likely are reproductively isolated. Phylogenetic analyses of nucleotide sequences of the rDNA internal transcribed spacer (ITS) region delineated both taxa, resolving *A. gillii* to a monophyletic clade strongly supported by bootstrap and Bayesian credibility values. Collectively, *A. gillii* and *A. nigrum* are well-defined morphologically and distinguishable molecularly, supporting the classification of these taxa at the specific rank.

KEY WORDS: dwarf mistletoe, hosts, MANOVA, *Pinus chihuahuana*, *Pinus leiophylla*, taxonomy

RESUMEN

Se obtuvieron datos adicionales morfológicos, fenológicos y moleculares para *Arceuthobium gillii* y *A. nigrum* en sus distribuciones geográficas del norte de México y Sur de Arizona. Se usó un análisis multivariante de varianza (MANOVA) y análisis de funciones discriminantes para demostrar que *A. gillii* and *A. nigrum* son diferentes morfológicamente. El análisis discriminante también indicó que las dimensiones del tercer internudo (longitud y ancho) y el diámetro basal de las plantas masculinas y femeninas contribuyeron en su mayoría a la clasificación y predicción de las especies. Se encontraron diferencias significativas en la altura de las plantas (femeninas y masculinas) así como en el color de la flor y las dimensiones del fruto entre estas dos especies de muérdago enano; *A. nigrum* presenta las dimensiones más grandes para cada uno de estos caracteres. Aunque supuestamente *A. nigrum* florece dos veces al año (primavera y otoño), nuestras observaciones indicaron que el periodo de antesis para este muérdago ocurre solamente una vez al año—otoño o inicios del invierno. En contraste, *A. gillii* florece en la primavera, sugiriendo que estos taxa se reproducen aisladamente. Un análisis filogenético de los espaciadores internos transcritos del ADN ribosomal separó a los dos grupos taxonómicos. *A. gilli* formó un grupo monofilético con buen soporte de bootstrap y valores de inferencia Bayesiana. En conclusión, la clasificación taxonómica de *A. gilli* y *A. nigrum* está bien sustentada con base en una buena definición de su morfología y en secuencias de ADN.

PALABRAS CLAVE: muérdago enano, huéspedes, MANOVA, *Pinus chihuahuana*, *Pinus leiophylla*, taxonomía

Gill's dwarf mistletoe, *Arceuthobium gillii* Hawksworth & Wiens (Santalales: Viscaceae), was described in 1964 from southern Arizona (Hawksworth & Wiens 1964). Previously, *A. gillii* was often misdiagnosed as *A. vaginatum* (Willdenow) Presl subsp. *cryptopodum* (Engelmann) Hawksworth & Wiens (southwestern dwarf mistletoe) or *A. divaricatum* Engelmann (pinyon pine dwarf mistletoe) in southeastern Arizona and northern Mexico, where these mistletoes are sympatric (Blumer 1910; Gill 1935; Hawksworth & Wiens 1964, 1972). *Arceuthobium gillii* can be readily distinguished morphologically and physiologically from the latter species by its shoot color and sexual dimorphism, highly glaucous fruits, host preferences, and time of anthesis (Hawksworth & Wiens 1964, 1996). Another similar dwarf mistletoe to *A. gillii* is *A. nigrum* Hawksworth & Wiens (black dwarf mistletoe) which was initially described as a subspecies of *A. gillii* (Hawksworth & Wiens 1965) as both taxa produce glaucous fruits, sexually dimorphic shoots, and share principal hosts in northern Mexi-

co—*Pinus leiophylla* Schiede ex Schlechtendal & Chamisso, *P. chihuahuana* Engelmann, and *P. lumholtzi* B.L. Robinson & Fernald. However, *A. nigrum* was raised to the specific rank by Hawksworth and Wiens (1989) based on morphological discontinuities with *A. gillii*; particularly, its larger and dark-green to black plants. Moreover, *A. nigrum* reportedly has two flowering periods (March–April and September–October; Hawksworth & Wiens 1989) while *A. gillii* flowers once annually (March–April; Hawksworth & Wiens 1996). Nevertheless, these species remain difficult to distinguish from each other particularly in high-elevation pine forests of central Durango, Mexico (Hawksworth & Wiens 1989, 1996; Mathiasen et al. 2008).

Because of the morphological similarities and host affinities of *A. gillii* and *A. nigrum* in northern Mexico, the geographic distribution of these dwarf mistletoes has remained unclear (Mathiasen et al. 2003, 2008, 2010). We, therefore, have been gathering additional morphological measurements and phenological observations since 1999 from Mexico as well as southern Arizona. According to previous work by Nickrent et al. (1994, 2004), *A. gillii* and *A. nigrum* can be readily distinguished molecularly via DNA analysis of the nuclear ribosomal internal transcribed spacer (nrITS) region. Therefore, we examined newly generated ITS sequences for *A. gillii* and *A. nigrum* to better assess species boundaries and the geographic distribution of these mistletoes in Mexico. Herein, we report the discriminatory morphological characters and phenology differences between *A. gillii* and *A. nigrum* as well as our nrITS analyses for both species across much of their geographic ranges.

METHODS

Morphological and Phenological Comparisons

To compare intra- and interspecific morphological characteristics of *A. gillii* and *A. nigrum*, we sampled 7 populations of *A. gillii* in southern Arizona and northern Mexico and 15 populations of *A. nigrum* from central and northern Mexico (Fig. 1). Plants were also collected and measured from the type localities (populations) for *A. gillii* and *A. nigrum* in Cochise County, Arizona, and Durango, Mexico, respectively (Hawksworth & Wiens 1964, 1965) (Fig. 1; locations 3 and 9). Ten to twenty male and female plants were collected and the dominant shoot from each plant was used for morphometric analyses. Plant characters measured were those used previously by Hawksworth and Wiens (1996) for the taxonomic classification of *Arceuthobium* spp.: (1) height, basal diameter, third internode length and width, and color of male and female plants; (2) mature fruit length, width, and color; (3) seed length, width, and color; (4) length and width of staminate spikes; (5) staminate flower diameters for 3- and 4-merous flowers as well as the length and width of petals; and, (6) anther diameter and distance from the petal tip. Each plant was measured less than 24-h after collection using a digital caliper, a dissecting microscope with a micrometer, or with a Bausch and Lomb 7× hand lens equipped with a micrometer. Staminate spike and flower measurements were made during peak anthesis, and fruit and seed measurements were made during the height of seed dispersal. Because the seasonal occurrence of flowering and seed dispersal for *A. gillii* and *A. nigrum* has received little attention (Hawksworth & Wiens 1996), phenological surveys for each taxon were conducted during the spring and fall of 1999, 2003, 2005, 2007, 2008, and 2010 as well as spring 2011.

A suite of multivariate and univariate statistical tests were utilized to assess species differences, collectively and separately, among the 20 morphological characteristics measured for *A. gillii* and *A. nigrum*. A one-way multivariate analysis of variance (MANOVA) was performed to control for experiment error (family-wise Type I error) and determine whether differences existed between the collective morphology of *A. gillii* and *A. nigrum* (Rancher 2002). One-way analysis of variance (ANOVA) was then used separately to examine the variance in the individual morphological characters of *A. gillii* and *A. nigrum*, and significant differences between means were determined using a posteriori contrast comparisons ($\alpha = 0.05$). Morphological data were also analyzed using discriminant function analysis (DFA; Quinn & Keough 2006) to determine how well each of the 20 morphological characteristics can be used to classify and predict species membership—*A. gillii* or *A. nigrum*. In addition, standardized discriminant function coefficients (DFC) were calculated to determine the relative importance of each morphological characteristic as a discriminator between species. Univariate and multivariate statistical analyses were performed using JMP 8 and JMP Pro 10 software (SAS Institute), respectively.

Fig. 1. Approximate locations of populations sampled for *Arceuthobium gillii* (open circles) in Arizona and Mexico and *A. nigrum* (dark circles) in Mexico. Localities from which plant material was collected for rDNA-ITS analysis (Fig. 4) are highlighted in bold. *A. gillii*: ARIZONA. **1** – Bear Canyon, Santa Catalina Mountains (RLM 1029, KC543492); **2** – Gardner Canyon, Santa Rita Mountains; **3** – 0.3 km E of the Reef, Huachuca Mountains (RLM 1027, KC543493); **4** – West Turkey Creek Campground, Chiricahua Mountains (RLM 1024, KC543494); **5** – Herb Martyr Picnic Area, Chiricahua Mountains (RLM 1023, KC543496); MEXICO. Chihuahua: **6** – 13 km E of Sonora state line on Route 16; Durango: **7** – 2 km E of La Quebrada. *A. nigrum*: MEXICO. Durango: **8** – 18 km W of Tepehuanes on rd. to Huacal; **9** – 50 km E of El Salto on Route 40 (RLM 0778, JQ723483); **10** – 11 km E of El Salto on Route 40 (RLM 0779, JC723481); **11** – 3 km E of El Salto on Route 40 (RLM 1105, KC543495); **12** – 23 km N of Route 40 on road to San Miguel de Cruces (RLM 0781, JQ723484) ; **13** – 102 km N of Route 40 on road to San Miguel de Cruces; **14** – 2 km N of Otinapa; **15** – 30 km W of Santiago Pasquiaro on road to Altares; Veracruz: **16** – Cofre de Perote 4 km S of Perote (RLM 0764, JQ723482); **17** – 3 km S of Sierra de Aqua on road to Pescados (RLM 1082, JQ723485); Puebla: **18** – 3 km NW of Los Hermanos; **19** – 6 km E of Route 119 on road to Tetla de Ocampo; Hidalgo: **20** – 5.5 km SW of Durango on Route 85 near Los Duraznos; **21** – 7 km NE of Metepec on road to Tenango de Doria.

PCR-amplification and Sequencing of the ITS region

Whole-genomic DNA was extracted from the aerial shoots of *A. gillii* (n = 4) and *A. nigrum* (n = 1) using a DNeasy® Plant Maxi Kit (Qiagen) and following the manufacturer's instructions; geographic location and voucher information are presented in Figure 1. Full-length, ITS sequences (partial 18S rRNA gene; complete ITS1, 5.8S rRNA gene, and ITS2; and, partial 26S rRNA gene) were PCR-amplified using the primer pair 18s 1830 for and 26S 40rev (Nickrent et al. 2004). PCR amplifications were performed in a total volume of 25 µL consisting of 2.5 µL 1X AccuPrime™ PCR buffer II (Invitrogen™; 600 mM Tris-SO_4 [pH 8.9], 180 mM $[NH_4]_2SO_4$, 20 mM $MgSO_4$, 2 mM dNTPs, thermostable AccuPrime™ protein, 10% glycerol), 0.5 µL (10 µM) of each primer (18s 1830 for and 26S 40rev), 0.1µL AccuPrime™ *Taq* DNA polymerase High Fidelity (Invitrogen™), 20.9 µL nuclease-free water (Promega), and 0.5 µL undiluted, genomic DNA (22–45 ng/µL). PCRs were performed using either a Eppendorf Mastercycler® Pro or Bio-Rad T100™ thermal cycler with identical cycling parameters: initial hold for 6 min. at 95°C; 5 cycles at 94°C for 30s, 55°C for 30s, and 72°C for 1 min.; 33 cycles at 94°C for 30s, 48°C for 30s, and 72°C for 1 min.; and, final extension step of 72°C for 10 min. Positive

controls and blank reactions (i.e., minus genomic DNA) were also included in each PCR run as checks for cross- and environmental contamination of template DNA, respectively.

Amplicon size for individual PCR products was checked via electrophoresis in 1.2% (wt/vol) agarose gels followed by ethidium bromide staining (1.2 mg/L 0.5X Tris-acetate-EDTA [TAE]) and visualization under ultra-violet fluorescence. PCR product(s) of *A. gillii* and *A. nigrum* were purified using the Wizard® Preps PCR DNA purification system (Promega) and the reagent ExoSAP-IT® (USB Inc.; 0.4 µL/µL of amplified DNA), respectively. Each amplicon was normalized (130 ng/reaction) and sequenced bidirectionally using an ABI 3730 DNA sequencer (Applied Biosystems), the referred primers (18s 1830 for and 26S 40rev), and a BigDye terminators DNA sequencing kit (Applied Biosystems).

Phylogenetic Analyses and Variability of the ITS

ITS sequences were assembled and edited using CodonCode Aligner (CodonCode Corporation), and confirmed as belonging to the genus *Arceuthobium* by BLASTN and comparison (nucleotide identity) to authenticated ITS sequences (Nickrent et al. 2004; Mathiasen et al. 2012). New DNA sequences generated for this study were deposited in GenBank (accession no. KC543492-KC543496). For phylogenetic analyses, a dataset was constructed consisting of the newly generated ITS sequences for *A. gillii* (KC543492-KC543494, KC543496) and *A. nigrum* (KC543495) as well as previously published sequences of each taxon (*A. gillii* L25689; *A. nigrum* AY288271, JQ723481-JQ723485, L25693) (Nickrent et al. 2004; Mathiasen et al. 2012). The dataset was then complemented with ITS sequences (outgroups) of *A. oaxacanum* (AY288273) and *A. yecorense* (AY288288) – sister taxa to *A. gillii* and *A. nigrum* (Nickrent et al. 2004). Sequences were aligned with the multiple sequence alignment option implemented in Clustal X v2.1 (Larkin et al. 2007), and the final alignment inspected in CodonCode Aligner. Maximum-likelihood (ML) analysis was performed using PAUP* 4.0b10 (Swofford 2003) and the best model of sequence evolution (Hasegawa-Kishino-Yano plus invariant sites [HKY+I]; Hasegawa et al. 1985) selected by the Akaike Information Criterion (AIC; Akaike 1974) as implemented in jModeltest 0.1.1 (Posada 2008). Likelihood settings corresponding to the HKY+I model were as follows: lset base = (0.3026 0.1618 0.2074), nst = 2, tratio = 2.5765, rates = equal, and pinvar = 0.7820. All nucleotides were included in the phylogenetic analysis and gaps were treated as missing characters. The heuristic search was performed with 1000 random-addition-sequence replicates (RAS), tree bisection-reconnection (TBR) branch swapping, and MULTREES in effect. Branch support was evaluated using 1000 bootstrap replicates and 100 RAS per pseudo-replicate.

Bayesian inference of phylogeny via the Markov Chain Monte Carlo (MCMC) method was also performed using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) as well as the best model and associated likelihood parameters executed in ML tree reconstruction. One cold and three heated Markov chain(s) were run, and samples were taken every 100 generations over 5.0×10^6 generations for a total of 50,000 sampled generations. The potential scale reduction factor (PSRF) for each of the model parameters was >1.0 when the program was terminated. Stationarity was accessed by examining the average standard deviations of split frequencies and likelihood values. Burn-in value (10%) was determined using Tracer v1.5 (Rambaut & Drummond 2009); removing 5000 burnin (sampled) generations. The remaining trees were utilized to calculate a 50% majority rule consensus tree and to determine the posterior probabilities.

To assess inter- and intraspecific variability of the ITS for *A. gillii* and *A. nigrum*, mean pairwise genetic distances (HKY+I) were calculated using the custom distance matrix option in PAUP*and the statistical software JMP^R Pro 10. Mean nucleotide difference (n) and the physical characteristics (substitution sites) of the ITS sequences within and between species were also assessed in Geneious R6 (Biomatters Ltd.) and CodonCode Aligner.

RESULTS AND DISCUSSION

Morphometry

Significant differences in morphology were found between *A. gillii* and *A. nigrum* (MANOVA, Wilks' Lambda = 0.00579 (20, 179) = 1536.34; $P<0.0001$) with the former consistently producing smaller plants/plant parts

(Table 1). Discriminant function analysis indicated that the most important morphological traits delineating *A. gillii* and *A. nigrum* were the third internodal length of female plants (DFC = -7.97), basal diameter of male (DFC = 5.53) and female plants (DFC = -5.03), and, the third internodal width of female (DFC = 3.95) and male plants (DFC = 3.81). Other morphological characteristics such as the diameter of 4-merous flowers (DFC = 2.51), female plant height (DFC= -2.29), staminate spike length (DFC = -2.13), male plant height (DFC = -2.13), seed length (DFC = -1.93), third internodal length of male plants (DFC = 1.68), and anther diameter (DFC = 1.0) also significantly contributed to the discrimination of *A. gillii* and *A. nigrum*. The remaining flower, fruit, and seed characteristics contributed the least to the discriminant function (DFC< |1.0|). Percent classification to the correct species using either a full-model (all 20 morphological characteristics) or reduced-model consisting of the five, most discriminative characteristics described above was 100% (200/200).

Examining the morphometric results, male plants of *A. gillii* (mean = 12.3 cm) were significantly shorter than those of *A. nigrum* (mean = 24.3 cm). Similarly, female plant height of *A. gillii* (mean = 14.2) was also significantly smaller than female plants of *A. nigrum* (mean = 19.6 cm)—approximately 5 cm shorter on average. The basal diameter and third internodal length and width of male and female plants, flower diameter (3- and 4-merous), and the dimensions of fruit and seeds were also significantly smaller for *A. gillii* when compared to *A. nigrum* (Table 1). Although mean staminate spike length was also significantly different between *A. gillii* (mean =15.3 mm) and *A. nigrum* (mean = 20.6 mm), mean staminate spike width was 2.9 mm regardless of species.

Our measurements of plant height for both mistletoe species were difficult to compare with those reportedly previously by Hawksworth and Wiens (1964, 1965, 1989, 1996) as they combined male and female plants in their analyses, and in lieu of reporting mean plant height for either species, they provided ranges and maximum measurements (Table 2). The maximum heights they reported for *A. gillii* (25 cm) and *A. nigrum* (45 cm) are similar to our maximum height for female plants of *A. gillii* (ca 28 cm) and for male plants of *A. nigrum* from central Mexico (ca. 53 cm; Table 1). Although the basal diameter for *A. gillii*, male and female plants combined, were approximately the same for our measurements (ca. 4.5 cm) as those reported previously by Hawksworth and Wiens (4.0 cm), the basal diameter of male and female plants of *A. nigrum* sampled in the present study were considerably larger (>7 mm) than that reported by Hawksworth and Wiens (5.0 mm). We also found that the dimensions of the third internode were generally larger for both species than those reported by Hawksworth and Wiens (see Tables 1 and 2 for comparison). Our measurements of 3-merous flowers for *A. gillii* and *A. nigrum*, however, were slightly smaller than those of Hawksworth and Wiens. Yet, to date and to the best of our knowledge, the diameters of 4-merous flowers, petal length and width as well as anther diameter of *A. gillii* have not been reported. Similarly, Mathiasen et al. (2012) as well as the present study remain the only reports to provide such measurement of 4-merous flowers, petal dimensions, and anther diameter for *A. nigrum*.

Our measurements of fruits were similar to those of Hawksworth and Wiens (1989, 1996) for the length of *A. nigrum* fruits, but our mean widths were larger (mean = 1.5; compare Tables 1 and 2). Our measurements indicated that *A. gillii* fruits are indeed larger (mean length = 5.8 mm, mean width = 3.6 mm) than what they reported; yet, seed length and width of *A. gillii* were similar in comparison to those found by Hawksworth and Wiens (compare Tables 1 and 2). As for *A. nigrum*, they reported larger seed lengths (3.5 mm) and smaller widths (1.3 mm) than our measurements (mean seed length = 3.1, mean seed width = 1.5 mm).

The only flower characteristic Hawksworth and Wiens reported for *A. nigrum* was the diameter of 3-merous flowers (3.5 mm), which was slightly larger than the average diameter for the 3-merous flowers we measured (mean = 3.2 mm). Our observations revealed that *A. nigrum* commonly produced 4-merous flowers as well; thus, we sampled and measured these flowers and found they averaged approximately 5 mm in diameter (mean = 4.8; Table 1). The mean diameter of 3- and 4-merous flowers of *A. nigrum* were also significantly larger than those of *A. gillii* (3-merous mean = 2.8 mm, 4-merous mean =3.6 mm). The petals of *A. nigrum* were relatively large in comparison to other dwarf mistletoes (Hawksworth & Wiens 1996; Mathiasen et al. 2012) as some lobes were >2 mm in length and nearly equally as large in width. In addition, examination of *A. nigrum* flowers also revealed that the adaxial surface of petals were dark red—a key phenotypic characteristic of this

TABLE 1. Morphological analyses (ANOVA) comparing *Arceuthobium gillii* and *A. nigrum*. Means followed by different capital letters in the same row were significantly different using a posteriori contrast comparisons (α = 0.05). Plant heights in cm and all other measurements in mm.

Character	ANOVA, Significance level	Mean (range) by Taxon	
		A. gillii	*A. nigrum*
Plant Height			
Male	$F_{1,228} = 138.00$, $P<0.0001$	12.3 A (7.3–21.6)	24.3 B (10.3–53.5)
Female	$F_{1,228} = 61.62$, $P<0.0001$	14.2 A (7.9–28.4)	19.6 B (9.3–37.2)
Basal Diameter			
Male	$F_{1,228} = 178.90$, $P<0.0001$	4.0 A (2.4–6.5)	7.0 B (4.4–12.5)
Female	$F_{1,228} = 338.97$, $P<0.0001$	5.3 A (2.9–8.5)	7.8 B (4.1–13.1)
Third Internode Length			
Male	$F_{1,228} = 175.50$, $P<0.0001$	10.6 A (6.1–16.7)	16.8 B (11.6–28.7)
Female	$F_{1,228} = 30.46$, $P<0.0001$	13.8 A (7.8–22.3)	16.5 B (11.8–31.8)
Third Internode Width			
Male	$F_{1,228} = 391.89$, $P<0.0001$	3.2 A (2.1–5.0)	4.9 B (4.0–7.8)
Female	$F_{1,228} = 102.49$, $P<0.0001$	4.2 A (2.6–6.6)	5.5 B (4.4–9.6)
Staminate Spike Length	$F_{1,398} = 100.84$, $P<0.0001$	15.3 A (6.9–25.6)	20.6 B (8.1–33.3)
Staminate Spike Width	$F_{1,398} = 1.51$, $P=0.2193$	2.9 A (2.1–3.5)	2.9 A (2.4–3.3)
Flower Diameter			
3-merous	$F_{1,198} = 103.71$, $P<0.0001$	2.8 A (2.1–3.5)	3.2 B (2.7–4.0)
4-merous	$F_{1,198} = 431.27$, $P<0.0001$	3.6 A (3.1–4.5)	4.8 B (3.6–5.4)
Petal length	$F_{1,398} = 236.72$, $P<0.0001$	1.4 A (1.0–1.8)	1.7 B (1.3–2.3)
Petal width	$F_{1,398} = 158.37$, $P<0.0001$	1.2 A (0.9–1.5)	1.4 B (0.8–1.9)
Anther Diameter	$F_{1,398} = 161.67$, $P<0.0001$	0.6 A (0.4–0.8)	0.8 B (0.5–1.1)
Anther Distance from Tip	$F_{1,398} = 124.10$, $P<0.0001$	0.4 A (0.2–0.7)	0.5 B (0.3–0.6)
Fruit Length	$F_{1,198} = 177.83$, $P<0.0001$	5.8 A (4.6–7.2)	6.9 B (5.2–8.8)
Fruit Width	$F_{1,198} = 118.42$, $P<0.0001$	3.6 A (2.8–4.4)	4.1 B (3.4–5.0)
Seed Length	$F_{1,198} = 97.60$, $P<0.0001$	2.8 A (2.0–3.3)	3.1 B (2.7–3.9)
Seed Width	$F_{1,198} = 55.82$, $P<0.0001$	1.4 A (1.1–1.6)	1.5 B (1.3–1.9)

TABLE 2. Morphological measurements for *Arceuthobium gillii* and *A. nigrum* reported by Hawksworth and Wiens (1996). Plant heights in cm and all other measurements in mm.

Character	*A. gillii*	*A. nigrum*
Plant Height[a]	8–15 [b] (Max. 25)	15–35 [b] (Max. 45)
Basal Diameter[a]	4.0 (2.5–8.0)	5.0 (3.0–8.0)
Mean Third Internode		
Length	10.7 (5.0–18.0)	10.8 (5.0–19.0)
Width	2.8 (2.0–4.5)	3.7 (2.5–6.0)
Plant Color	Greenish-brown	Dark brown-green, dark brown, black
Mean Flower Diameter[c]	3.2	3.5
(3-merous flowers)		
Anthesis	March–April	March–April, September–October
Seed Dispersal	October	September–October
Fruit		
Length	4.0–5.0 [b]	7.0 (6.0–9.0)
Width	2.0–3.0 [b]	3.5
Seed		
Length	3.1	3.5
Width	1.4	1.3

[a] Male and female plants combined.

[b] No mean was provided by Hawksworth and Wiens (1996), only a range.

[c] No other flower measurements (e.g., 4-merous, anther diameter, etc.) were reported by Hawksworth and Wiens (1996).

species throughout its geographic range (Fig. 2)—and remarkably different compared to the green petals of *A. gillii* (Fig. 3). These conspicuous, red-colored petals of *A. nigrum*, however, were never reported in the literature until recently by Mathiasen et al. (2012). The reason(s) for why this discriminatory and diagnostically useful

Fig. 2. Staminate plants of *Arceuthobium nigrum* in October in Durango, Mexico. Note the dark red flowers with 3 or 4 perianth lobes.

characteristic has gone unnoticed for nearly a half-century likely will remain unknown. However, petal color—a phenotypic characteristic unaffected by plant age—should be utilized by field botanists and forest health specialist alike for differentiating *A. nigrum* and *A. gillii*.

Phenology

According to our observations, flower phenology (period of anthesis) appeared also to be a delineating characteristic between *A. gillii* and *A. nigrum*. In southern Arizona and Chihuahua, Mexico, *A. gillii* flowered in early-March, or as late as May in some years, and terminated by late-April. In contrast, *A. nigrum* flowered only once annually between September and January. Hawksworth and Wiens (1989, 1996) reported two flowering periods—March–April and September–October—for *A. nigrum*; however, according to our records and review of herbarium specimens, a spring flowering period for this dwarf mistletoe has never been well documented. We, therefore, examined numerous populations of *A. nigrum* with male plants, including the type-locality in Durango, Mexico, in mid- to late-March 2003, 2005, and 2007, and again in early-April 2011; no open, staminate flowers were evident. Moreover, within the same localities and time periods, staminate flowers were not approaching maturity. Periodic fall surveys of the same *A. nigrum* populations in 1999–2010, however, revealed that this dwarf mistletoe flowered starting in mid-September and continued into November in Durango, Mexico, and peak flowering occurred in early-October. Similarly, anthesis began slightly later (mid-September) in central Mexico and continued into January; yet, the period of peak anthesis within central Mexico has remained uncertain. As with the advent of flowering, our observations indicated that *A. nigrum* began dispersing seed in early-September, peaked in mid-October, and continued into mid-November in Durango as well as

Fig. 3. Staminate plants of *Arceuthobum gillii* in late-March in southern Arizona. Note the green flowers with 3 perianth lobes.

central Mexico. Likewise, seed dispersal by *A. gillii* initiated in early September and peaked in early-October. These phenological observations for seed dispersal of *A. gillii* and *A. nigrum* were consistent with those previously reported (Hawksworth & Wiens 1996); however, they were not taxonomically informative between species.

Molecular Identification

Phylogenetic analyses of the ITS region for *A. gillii* and *A. nigrum* supported the morphological data, effectively delineating species as all plants diagnosed morphologically as *A. gillii* resolved to a monophyletic group (Fig. 4). The size of the ITS region (ITS1-5.8S-ITS2) for each sequence of *A. gillii* and *A. nigrum* was 600 bp while the total amplicon per taxon consisted of 623 bp with a partial sequence of the 18S (4 bp) and 26S (19 bp) located at the 5′- and 3′-end, respectively. The mean interspecific genetic distance (HKY+I) for both *A. gillii* (n = 10 pairwise comparisons) and *A. nigrum* (n = 21 pairwise comparisons) was 0.0018 substitutions/site. Similarly, mean nucleotide divergence (n) within species for *A. gillii* as well as *A. nigrum* was 1.0 nucleotide. Although readily distinguishable via DNA analysis, our results suggested that *A. nigrum* and *A. gillii* are more closely-related phylogenetically than previously demonstrated by Nickrent et al. (2004). The alignment used for ML and Bayesian analyses—including outgroup taxa *A. yecorense* and *A. oxacanum* as well as sequences of *A. gillii* and *A. nigrum* utilized in Nickrent et al. 2004—consisted of 625 total characters of which 531 were constant, 38 variable and parsimony-uninformative, and 56 parsimony-informative. The combined ML and Bayesian consensus tree strongly supported a single-clade (common ancestry; bootstrap value 100%, posterior probability 1.00) consisting of *A. nigrum* and *A. gillii*, with the latter resolving to a more distantly related, monophyletic subclade (bootstrap value 94%; posterior probability 1.00). The mean genetic distance (HKY+I; n = 49 pairwise

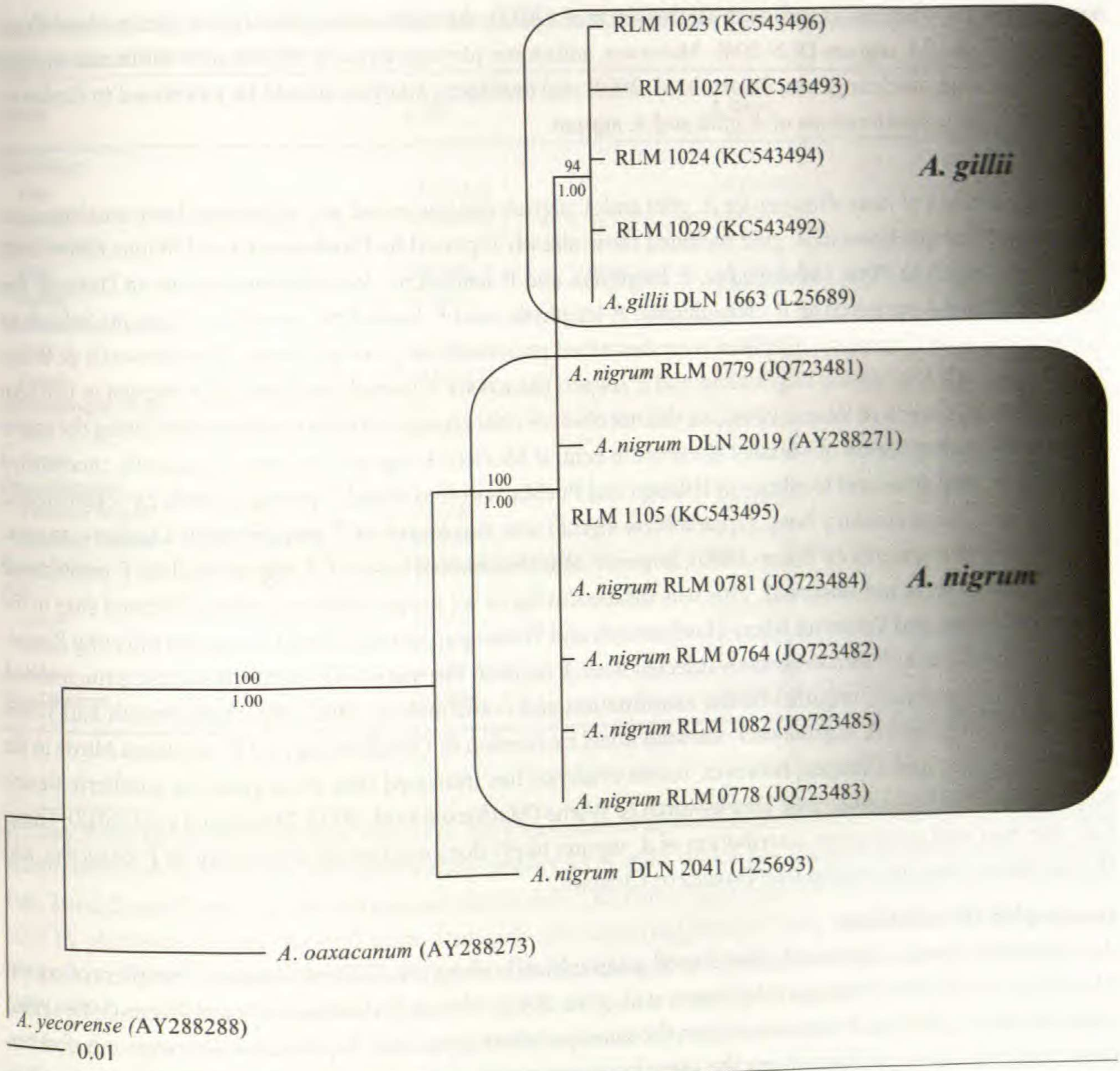

Fig. 4. Phylogram inferred from maximum likelihood (ML) analysis of nuclear ribosomal ITS sequences of *Arceuthobium gillii* and *A. nigrum*, and the outgroup taxa, *A. yecorense* and *A. oaxacanum* using a HKY+I model of sequence evolution. Numbers above branches indicate ML bootstrap values >70% (after 10^3 replicates), numbers below are Bayesian posterior probabilities > 0.95 (after 5.0×10^6 generations). Collector abbreviations RLM and DLN are for Robert L. Mathiasen and Daniel L. Nickrent, respectively, and precede assigned voucher numbers. GenBank accession numbers for each sequence are provided in parentheses (GenBank Accession no.). The geographical origin of *A. gillii* and *A. nigrum* collections/accessions are provided in Fig. 1 (bold).

comparisons) and mean nucleotide divergence (n) between *A. gillii* and *A. nigrum* was 0.0083 substitutions/site and 4.9 nucleotides, respectively. Forty-eight of the forty-nine (48/49) pairwise comparisons between *A. gillii* and *A. nigrum* shared 100% nucleotide identity (207/207 bases) in the ITS1 and were separated by approximately five or fewer substitutions across the 5.8S rRNA gene and ITS2—notably, consistent A/G nucleotide changes at positions 335, 421, 457, and 535. In contrast, the mean genetic distance (HKY+I; n = 4 pairwise comparisons) and nucleotide divergence between our newly generated ITS sequences of *A. gillii* and *A. nigrum* DLN 2041 sequence (GenBank L25693) utilized by Nickrent and colleagues (Fig.2 in Nickrent et al. 2004) was 0.0336 substitutions/site and 19.3 nucleotides, respectively. These genetic comparisons between *A. gillii* and that of *A. nigrum* DLN 2041 were nearly equal to those calculated in the present study when comparing the interspecific genetic variability of outgroup taxa, *A. yecorense* and *A. oaxacanum* (0.0331 substitutions/site, 19 nucleotide difference). The identity of *A. nigrum* DLN 2041 – species or potential hybrid—remains unclear and currently is being assessed (S.C. Kenaley & R.L. Mathiasen, unpubl. data). Therefore, the ITS sequences of *A.*

nigrum presented herein, as well as in Mathiasen et al. (2012), should be utilized for future species-level identification in lieu of *A. nigrum* DLN 2041. Moreover, additional phylogenetically informative molecular markers (e.g., single-copy nuclear genes; Duarte et al. 2010) and multigene analysis should be examined to further resolve the genetic relationships of *A. gillii* and *A. nigrum*.

Hosts

Our examination of host affinities for *A. gillii* and *A. nigrum* did not reveal any additional host-mistletoe combinations. Principal hosts of *A. gillii* included those already reported by Hawksworth and Wiens (1964, 1965, 1989, 1996) such as *Pinus chihuahuana*, *P. leiophylla*, and *P. lumholtzii*. *Arceuthobium nigrum* in Durango was most often found parasitizing *P. chihuahuana*, *P. leiophylla*, and *P. lumholtzii*, as well as *P. teocote* Schiede ex Schlechtendal & Chamisso; all pines were described previously as principal hosts (Hawksworth & Wiens 1996). Although *P. arizonica* Engelmann and *P. cooperi* Blanco are reported rare hosts of *A. nigrum* in northern Mexico (Hawksworth & Wiens 1996), we did not observe either host-mistletoe combination during the course of our work in that region of Mexico. However in central Mexico, *A. nigrum* was most frequently encountered on *P. teocote*, and at several localities in Hildago and Puebla, we often found *P. patula* Schiede ex Schlechtendal & Chamisso as a secondary host. *Arceuthobium nigrum* was also found on *P. pseudostrobus* Lindley – an occasional host (Hawksworth & Wiens 1996); however, other occasional hosts of *A. nigrum* such as *P. montezumae* A. B. Lambert, were not observed. This was disappointing as we purposefully examined forested sites in the States of Hidalgo and Veracruz where Hawksworth and Wiens purportedly found *A. nigrum* infecting *P. montezumae*, finding only *P. teocote* severely infected with *A. nigrum*. The status of *P. montezumae* as a principal host of *A. nigrum*, therefore, requires future examination and confirmation. Similarly, Hawksworth and Wiens (1989, 1996) reported *A. nigrum* on *P. lawsonii* Roezl ex Gordon & Glendinning and *P. oaxacana* Mirov in the States of Oaxaca and Chiapas; however, recent evidence has indicated that these pines in southern Mexico were infected with *A. hondurense* Hawksworth & Wiens (Mathiasen et al. 2003; Mathiasen et al. 2012). Therefore, the host and geographic distribution of *A. nigrum* likely does not include *P. lawsonii* or *P. oaxacana*, and this mistletoe does not extend into Oaxaca or Chiapas.

Geographic Distributions

Arceuthobium nigrum is primarily distributed geographically along the Central Volcanic Cordillera of central Mexico and north into Durango (Mathiasen et al. 2010, 2012). Although Hawksworth and Wiens (1989, 1996) reported that *A. gillii* and *A. nigrum* occur in the same mountain range near Tepehuanes, Durango, our observations and collections of plants from the same locations examined by Hawksworth in 1987 (Hawksworth & Wiens 1996), did not support this report. All of the populations we examined in the Tepehuanes area represented *A. nigrum*. The closest, and thus far, only population of *A. gillii* we found to date in Durango was near La Quebrada immediately south of the border with the State of Chihuahua (Fig. 1, location 7). Therefore, the common dwarf mistletoe on *P. leiophylla* and *P. chihuahuana* in Durango is *A. nigrum*, while *A. gillii* was the most frequent dwarf mistletoe on these pines in Chihuahua; we have not observed *A. nigrum* in Chihuahua thus far. Likewise, although *A. gillii* may be found to be sympatric with *A. nigrum* in Durango, we have not observed these mistletoes in the same pine stand or general location.

SUMMARY

The principal morphological and phenological differences distinguishing *A. gillii* from *A. nigrum* are summarized in Table 3. In general, the plants, flowers, and fruits of *A. gillii* are significantly smaller than those of *A. nigrum*, but their plant color variations and habit are very similar, making it difficult to discriminant them *in situ*. Although male plants of *A. gillii* are often green (Fig. 3), plants of both sexes are typically dark brown to greenish-brown (Fig. 2), and we have observed female plants of both species that appear almost black. Both species have relatively wide staminate spikes (ca. 3.0 mm) and demonstrate a striking sexual dimorphism: female plants being densely branched and compact and male plants are open and spreading (see Figs. 3.1 and 3.2 in Hawksworth & Wiens 1996). Furthermore, both species have strikingly glaucous fruits and they parasitize

TABLE 3. Principal morphological and phenological characteristics distinguishing *Arceuthobium gillii* and *A. nigrum*. Mean plant heights in cm and all other means in mm; maximum size measured in parentheses.

Character	*A. gillii*	*A. nigrum*
Plant Height		
Male	12.3 (21.6)	24.3 (53.5)
Female	14.2 (28.4)	19.6 (37.2)
Basal Diameter		
Male	4.0 (6.5)	7.0 (12.5)
Female	5.3 (8.5)	7.8 (13.1)
Third Internode Length		
Male	10.6 (16.7)	16.8 (28.7)
Female	13.8 (22.3)	16.5 (31.8)
Third Internode Width		
Male	3.2 (5.0)	4.9 (7.8)
Female	4.2 (6.6)	5.5 (9.6)
Flower Diameter		
3-merous flowers	2.8 (3.5)	3.2 (4.0)
4-merous flowers	3.6 (4.5)	4.8 (5.3)
Flower Color	Green or Green-yellow	Red
Fruit		
Length	5.8 (7.2)	6.9 (8.8)
Width	3.6 (4.4)	4.1 (5.0)
Anthesis	March–April	September–January
Seed Dispersal	October	September–October

the same hosts in northern Mexico. However, the mean sizes of all the morphological characters we examined, except staminate spike width, are significantly smaller for *A. gillii*, which has green, not red flowers (compare Figs. 2 and 3), and flowers in the spring and not the fall. Therefore, these taxa can be distinguished from each other by plant size, flower size and color, fruit size, and flowering period (Table 3) and should be treated as distinct species. Difficulties with identifying them in the field will be related primarily to their similar growth habit and plant color, so it is necessary to examine many plants from a population, determine plant, flower, and fruit dimensions for at least a small sample of these parameters and observe if male plants are flowering. If male plants are flowering, then the color of the flowers (green or red) and season (spring or fall) will positively identify if the population represents *A. gillii* or *A. nigrum* in northern Mexico where these species may co-occur. Based on our current knowledge of their distributions, *A. gillii* only occurs from southern Arizona and New Mexico south to far northern Durango, Mexico (Fig. 1), and *A. nigrum* occurs from central Durango near Tepehuanes south as far as Puebla, Tlaxcala, and Veracruz (Mathiasen et al. 2010, 2012). Therefore, it should be noted that most of the populations of *A. gillii* reported by Mathiasen et al. (2008) based on herbaria records from southern and central Durango probably represent populations of *A. nigrum*.

ACKNOWLEDGMENTS

We would like to thank Brian P. Reif for performing the DNA extractions described herein. We also sincerely appreciate the help of Gustavo Perez and Jeremy R. Thompson with the Spanish translation for the Resumen. Reviews of the manuscript by Socorro González Elizondo and Greg Filip are sincerely appreciated and helped to greatly improve the paper.

REFERENCES

AKAIKE, H. 1974. A new look at the statistical model identification. IEEE Trans. Autom. Contr. 19:716–723.

BLUMER, J.C. 1910. Mistletoe in the Southwest. Pl. World 13:240–246.

DUARTE, J.M., P.K. WALL, P.P. EDGER, L.L. LANDHERR, H. MA, J.C. PIRES, J. LEEBENS-MACK, AND C.W. DEPAMPHILIS. 2010. Identification of

shared single copy nuclear genes in *Arabidopsis*, *Populus*, *Vitis* and *Oryza* and their phylogenetic utility across various taxonomic levels. BMC Evol. Biol. 10:61.

Hawksworth, F.G. and D. Wiens. 1964. A new species of *Arceuthobium* from Arizona. Brittonia 16:54–57.

Hawksworth, F.G. and D. Wiens. 1965. *Arceuthobium* in Mexico. Brittonia 17:213–238.

Hawksworth, F.G. and D. Wiens. 1972. Biology and classification of dwarf mistletoes (*Arceuthobium*). Agriculture Handbook 401, USDA Forest Service, Washington, D.C.

Hawksworth, F.G. and D. Wiens. 1989. Two new species, nomenclatural changes, and range extensions in Mexican *Arceuthobium* (Viscaceae). Phytologia 66:5–11.

Hawksworth, F.G. and D. Wiens. 1996. Dwarf mistletoes: biology, pathology, and systematics. Agriculture Handbook 709, USDA Forest Service, Washington, D.C.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Molec. Evol. 22:160–174.

Huelsenbeck, J.P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinform. Appl. Note 17: 754–755.

Larkin, M.A., G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, T.J. Gibson, and D.G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinform. 23:2947–2948.

Mathiasen, R.L., C.M. Daugherty, and V. Guerra De la Cruz. 2010. Muerdagos enanos (*Arceuthobium* spp.) en el sur de Mexico: Distribucion, hospederos y cambios en la nomenclatura. In: Proceedings of the XV Simposio Nacional de Parasitologia Forestal, November 18–20, 2009, Oaxaca, Mexico. Pp. 160–166.

Mathiasen, R.L., M.C. González Elizondo, M. González Elizondo, B.E. Howell, I.L. López EnriqueZ, J. Scott, and J.A. Tena Flores. 2008. Distribution of dwarf mistletoes (*Arceuthobium* spp., Viscaceae) in Durango, Mexico. Madroño 55:161–169.

Mathiasen, R.L., S.C. Kenaley, and B.P. Reif. 2012. Distribution and morphological characteristics of *Arceuthobium hondurense* and *A. nigrum* (Viscaceae) in Mexico. J. Bot. Res. Inst. Texas 6: 599–609.

Mathiasen, R.L., J. Melgar, J. Beatty, C. Parks, D.L. Nickrent, S. Sesnie, C. Daugherty, B. Howell, and G. Garnett. 2003. New distributions and hosts for mistletoes parasitizing pines in southern Mexico and Central America. Madroño 50:115–121.

Nickrent, D.L., M.A. García, M.P. Martín, and R.L. Mathiasen. 2004. A phylogeny of all species of *Arceuthobium* (Viscaceae) using nuclear and chloroplast DNA sequences. Amer. J. Bot. 91:125–138.

Nickrent, D.L., K.P. Schuette, and E.M. Starr. 1994. A molecular phylogeny of *Arceuthobium* (Viscaceae) based on nuclear ribosomal DNA internal transcribed spacer sequences. Amer. J. Bot. 81:1149–1160.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Molec. Biol. Evol. 25:1253–1256.

Quinn, G.P. and M.J. Keough. 2006. Experimental design and data analysis for biologists. Cambridge Univ. Press, United Kingdom.

Rambaut, A. and J. Drummond. 2009. Tracer 1.5, 2009. MCMC Trace File Analyser. http://tree.bio.ed.ac.uk/software/tracer/.

Rancher, A.C. 2002. Methods of multivariate analysis. 2nd edition. John Wiley and Sons, Inc., New York, New York.

Swofford, D.L. 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, USA.